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(54) Title: NEEDLELESS ADMINISTRATION OF POLYNUCLEOTIDE FORMULATIONS

(57) **Abstract**

This invention relates to a method of inducing an immune response to protein antigen expressed *in vivo* through the use of a needleless jet injection device for the administration of DNA, DNA formulations and/or other polynucleotides for genetic vaccination, gene delivery, and gene therapy.

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TITLE OF THE INVENTION
NEEDLELESS ADMINISTRATION OF POLYNUCLEOTIDE FORMULATIONS.

CROSS-REFERENCE TO RELATED APPLICATIONS

Not Applicable

STATEMENT REGARDING FEDERALLY-SPONSORED R&D

Not Applicable

REFERENCE TO MICROFICHE APPENDIX

Not Applicable

FIELD OF THE INVENTION

This invention relates to the use of a needleless jet injection device for the administration of polynucleotide and polynucleotide formulations for genetic vaccination, gene delivery, and gene therapy.

BACKGROUND OF THE INVENTION

Traditionally, injection of medication or vaccine formulations into humans has been via syringe/needle combinations. This is an effective means of delivery, however, the risks associated with needles (e.g., the transmission of blood-borne pathogens) are significant. Moreover, needles provide the additional burden of needlestick injury which is a nuisance especially in young children. With the advent of advanced needleless injector devices such as Bioject's BIOJECTOR™ 2000, the possibility of injecting materials intramuscularly with greater effectiveness, less sustained damage to the skin and a decreased risk of transmission of blood-borne pathogens was realized; see U.S. Patent Nos. 5,383,851, 5,064,413, 4,940,460, 4,790,824, and 4,596,556.

Experiments were done using jet injection technology (specifically a PED-O-JET™ injector) to introduce reporter genes, for example, chloramphenicol acteyl thransferase (CAT) to assess the effectiveness of this system for gene transfer; see Furth et al., 1992 *Analytical Biochem.* 205:365-368. CAT activity, however, could not be detected in all samples and, further,

the samples exhibited variability. The use of such technology in generating an immune response was not tested.

Experiments were later carried out in the area of DNA-based immunization. Plasmid DNA encoding hepatitis B virus surface antigen was injected into mature muscle cells with the intent of delivering the DNA using the BIOJECTOR™ needleless jet injection system; see Davis et al., 1994 *Vaccine* 12(16):1503-1507. Levels approaching 4-fold greater antibody production were obtained as opposed to the traditional syringe/needle combination, yet when compared with the levels obtained with regenerating muscle cells, the increase was not as substantial. Further, these increases were not observed in rats.

Intradermal administration via a needleless injector device was attempted with a synthetic gene encoding the G protein of bovine respiratory syncytial virus (BRSV) and the resulting data compared with that obtained via a needle administration; Schrijver et al., 1998 *Vaccine* 16(2/3):130-134. After three immunizations into calves, antibody titres with the needleless injections were significantly higher.

Intramuscular injections are often the most effective means of inducing an immune response. Thus, it is desired that results akin to that obtained intradermally be obtained intramuscularly. In order to effectively use the needleless injection device to induce such an immune response intramuscularly in humans, more consistency and a greater induction of the immune system is needed. Thus, it would be desirable to identify a system that could achieve efficient transfer of polynucleotide formulations such as DNA consistently and induce stronger immune responses than that achieved with conventional needle injection into muscle.

SUMMARY OF THE INVENTION

This invention relates to a method of inducing an immune response to protein antigen expressed *in vivo* through the use of a needleless jet injection device for the administration of DNA, DNA formulations and/or other polynucleotides for genetic vaccination, gene delivery, and gene therapy.

This invention further relates to an improved method of inducing an immune response to a polynucleotide vaccine in an animal, preferably a human, using a needleless injector comprising introducing the vaccine

intramuscularly wherein the polynucleotide vaccine, upon entry into the animal, is not present in a bolus, and wherein the polynucleotide vaccine, upon entry into the animal, is substantially evenly distributed in an area of muscle tissue.

This invention further relates to a method of inducing an potent immune response to a polynucleotide vaccine in an animal using a needleless injector comprising introducing the vaccine intramuscularly wherein the polynucleotide vaccine, upon entry into the animal, is not present in a bolus, the polynucleotide vaccine having spread laterally along structural tissue planes have little resistance to fluid flow, wherein the polynucleotide vaccine, upon entry into the animal, is substantially evenly distributed in an area of muscle tissue, and wherein the introduction of the vaccine is controlled by passing the vaccine through an orifice having a predetermined diameter.

In a preferred embodiment, the nucleic acid is a DNA or cDNA. Particularly preferred embodiments of this invention are to the delivery of DNA which is either naked, complexed or viral. Especially preferred embodiments comprise synthetic DNA molecules encoding HIV *gag* and synthetic DNA molecules encoding modified forms of HIV *gag*.

This invention also relates to a method of delivering DNA to an animal for purposes of gene therapy using a needleless injector comprising introducing the vaccine intramuscularly wherein the polynucleotide vaccine, upon entry into the animal, is not present in a bolus, and wherein the polynucleotide vaccine, upon entry into the animal, is substantially evenly distributed in an area of muscle tissue. Preferably, the amount is sufficient to effect the desired prophylactic or therapeutic response.

Administration by the method of this invention results in significant enhancements in antibody titers (i.e., in some instances over 100-fold) compared to conventional needle injections. The method of this invention also produces a marked reduction in variability from animal to animal, and increased rates of sero-conversion (near 100%). This constitutes a surprising and significant advance over the traditional administration (i.e., via a syringe/needle combination), which leads to only a moderate response with moderate rates of sero-conversion (~40%) at best. The administration of polynucleotides by needless jet injection results in a substantially increased distribution of injected fluid within the muscle compared to conventional

needle/syringe delivery. It appears that the jet of polynucleotide formulation, upon entry into the body, travels laterally to the axis of injection as it traverses structural planes of tissue like fascia. These planes tend to have a decreased level of resistance, so the liquid jet of vaccine has a tendency to travel along them. While not wishing to be bound by theory, the increased distribution of polynucleotide in the muscle is probably required to induce the improved immune response. Several theories could explain how the increased distribution improves the immune response. The increased distribution of vaccine throughout the muscle could result in more antigen presenting cells such as macrophages and dendritic cells taking up DNA since these cells are found at low density within muscle tissue. DNA uptake by antigen presenting cells is believed to be an extremely potent means of inducing immune responses. Alternatively, forcing liquid into the muscle and connective tissue might induce an inflammatory response which would attract a variety of immune cells such as macrophages, dendritic cells, eosiniphils, neutrophils, etc. which could result in increased uptake of either DNA or of expressed antigen and hence lead to increased immunogenicity. Moreover, the variability in measured antibody response of the cohorts immunized with the needleless injector is significantly reduced relative to that of the syringe/needle.

In a preferred embodiment, the injectate is DNA. Especially preferred embodiments include the delivery of DNA which is naked, complexed or viral. DNA may be in plasmid form, either supercoiled or not. Especially preferred are synthetic DNA molecules which encode HIV *gag* and synthetic DNA molecules encoding modified forms of HIV *gag, env, pol, nef, or rev* genes, influenza HA, M1, or NP genes, and herpes simplex virus (HSV) proteins such as gD and gB or papillomavirus L1 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 illustrates the results of the gel quantification of the component of supercoiled DNA (SC) to total DNA (SC + OC) after various treatments with needle or needleless injector; see Example 2B for details.

FIGURES 2A and 2B illustrates a gel (Fig. 2A) and the results of gel quantification (Fig. 2B) wherein samples at two different concentrations were passed through either a No.2 (small diameter orifice) or No.5 (large

diameter orifice) BIOJECTOR™ syringe tip, either slowly by hand in a drop-wise fashion or expelled by the burst of CO_2 gas from the BIOJECTOR™; see Example 2C for details.

FIGURES 3A and 3B illustrates a gel (Fig. 3A) and the results of gel quantitation (Fig. 3B) wherein samples at a high concentration passed through either a No.2 (small diameter orifice) or No.5 (large diameter orifice) BIOJECTOR™ syringe tip, either slowly by hand in a drop-wise fashion or expelled by the burst of CO_2 gas from the BIOJECTOR™; see Example 2D for details.

FIGURE 4 (A) illustrates the haemagglutination inhibition (HI) responses of groups of four African green monkeys immunized by needle or BIOJECTOR™ three times with 10 μg of influenza HA DNA per immunization; see Example 4A for details.

FIGURE 4 (B) illustrates ELISA antibody responses for groups of four African green monkeys immunized by needle or BIOJECTOR™ three times with 10 μg of HA DNA per immunization; see Example 4A for details.

FIGURE 5 (A) illustrates enhanced anti HIV-1 gag antibody titers (as measured by ELISA) in guinea pigs immunized with the BIOJECTOR™ as compared to that obtained with a needle four weeks post a single dose of either 80, 400 or 2000 mcg of HIV-1 gag DNA; see Example 4B for details.

FIGURE 5 (B) illustrates the Anti-gag antibody responses four weeks after a second immunization with the identical vaccines in the fourth week; see Example 4B for details.

FIGURE 6 illustrates the results after cohorts of five guinea pigs were immunized at either upper or lower hamstring sites using either a needle or BIOJECTOR™ needleless syringe, with or without anesthesia; see Example 4C for details.

FIGURE 7 illustrates the results from the reporter gene activity of human heat resistant Secreted Alkaline Phosphatase (SeAP) measured in guinea pigs injected once with either a high or low dose of SeAP encoding plasmid DNA, either by insulin syringe or BIOJECTOR™. The cohort averages are plotted as a function of time; see Example 5A for details.

FIGURES 8A, B and C illustrate the results from the reporter gene activity of human heat resistant secreted Alkaline phosphatase (SeAP)

measured in guinea pigs injected once with either a high or low dose of SeAP encoding plasmid DNA, either by insulin syringe or Biojector™. The individual animals values are plotted as a function of time day 2 is Fig. 8A; day 4 is Fig. 8B; and day 7 is Fig. 8C; see Example 5A for details.

FIGURE 9 illustrates the results from viscosity measurements of DNA in either saline or saline/glycerol at various temperatures and concentrations of DNA; see Example 2E for details.

DETAILED DESCRIPTION OF THE INVENTION

As used throughout the specification and claims, the following definitions apply:

"Complexed" means interacting to other matter via physical forces at close range, including but not limited to, electrostatic, van der Waals, hydrophobic and double layer, to associate or continue to interact for a time sufficient to achieve the desired function. Desired functions include but are not limited to protection from nucleases, transfection of the cell wall, endocytosis through receptor mediated pathways as well as others.

"Polynucleotide" means a nucleic acid which contains essential regulatory elements such that upon introduction into a living, vertebrate cell, it is able to direct the cellular machinery to produce translation products encoded by the genes comprising the polynucleotide.

"PNV" is the designation given a polynucleotide vaccine.

"Promoter" refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers."

"Leader" refers to a DNA sequence at the 5' end of a structural gene which is transcribed along with the gene. The leader usually results in the protein having an N-terminal peptide extension sometimes called a pro-sequence. For proteins destined for either secretion to the extracellular medium or a membrane, this signal sequence, which is generally hydrophobic, directs the protein into endoplasmic reticulum from which it is discharged to the appropriate destination.

"Intron" refers to a section of DNA occurring within a gene which does not code for an amino acid in the gene product. The precursor RNA of the intron is excised and is therefore not transcribed into mRNA nor translated into protein.

"Restriction site" refers to a sequence specific cleavage site of restriction endonucleases.

"Vector" refers to some means by which DNA fragments can be introduced into a host organism or host tissue. There are various types of vectors including plasmid, bacteriophages and cosmids.

"Effective amount" means sufficient PNV is injected to produce adequate levels of the transgene product. One skilled in the art recognizes that this level will vary depending on therapeutic or immuno- function desired.

"Effective dose" is an amount sufficient to produce adequate immune response in the subject to afford a protective immune response.

"Geometric Mean Titer" or "GMT" means the nth root of the product of n variables.

"Bolus" refers to a roughly spherical accumulation of injectate, containing substantially the entire dose of the vaccine.

One aspect of the present invention is that the needleless delivery system results in improved immunogenicity of greater than two orders of magnitude larger than naked DNA in saline or as large as over 100-fold enhancement over similar vaccination via a syringe/needle combination have been obtained after only two injections. There is also observed a near 100% seroconversion and reduced variability amongst the samples. In Fig.5(B), the highest antibody titers measured in an animal from a needleless injection cohort is at most 64-fold higher than the lowest titer measured, whereas the highest titer measured in a needle/syringe cohort is as much as 1,024-fold higher than the lowest titer.

An aspect deemed important in the present invention is use of the proper syringe tip in conjenction with the needleless injectin device. If the orifice of the syringe tip is too small or presents too large of a viscous resistance or impeadance, then the vaccine will not be injected intramuscularly. Rather, it will only penetrate the dermis, and will be present in substantially a subcutaneous bolus. Conversely, if the syringe tip is too large, or presents too small a viscous resistance or impeadance to flow, then the jet of the vaccine

will penetrate too deeply, cutting a jet track in the muscle as it traverses, often striking bone or other deep tissues.

Another aspect of the present invention is the discovery that, at higher concentration (e.g., at 5 milligrams/ml), the nucleic acid is better protected from shear forces and is less susceptible to degradation. It has been found that there is a higher percentage of available DNA at 5 mg/ml than that found at 1 mg/ml). Therefore, one method of increasing the potency of the vaccine is to increase the concentration of polynucleotide present. This is unexpected over the prior art and further contributes to the novelty of the present invention. Thus, the present invention further entails introducing the vaccine, or DNA in the case of DNA delivery, at a concentration of at least 5 mg/ml.

Preferred embodiments of this invention are to the delivery of polynucleotides which are either naked, complexed or viral, with the polynucleotides being DNA or RNA. Essentially, any polynucleotide formulation injectable by syringe can be alternatively injected using a needleless injection device, leading to increased and enhanced biodistribution. Preferably, the administration of the polynucleotide formulation is via Bioject's BIOINJECTOR TM 2000 injection device, although other systems can be used.

The DNA or other polynucleotide chosen can be inserted into an appropriate expression vector. The vector may be any known vector, including plasmids, cosmids and viral vectors which can function in the recipient cell. Typically, the vectors used in any of the host cells will contain a 5' flanking sequence (also referred to as a "promoter"), whether it be a native (i.e., from the same species and/or strain as the host cell), heterologous (i.e., from a different species and/or strain), or a hybrid thereof. Preferably, the promoter is that of cytomegalovirus (CMV). Optionally, the vectors may also contain other expression-control elements, such as enhancers and sequences which assist the host in expressing the peptide, such as an origin of replication element, a transcriptional termination element, a complete intron sequence containing a donor and acceptor splice site, a signal peptide sequence, a ribosome binding site element, a polyadenylation sequence, a polylinker region for inserting the nucleic acid encoding the polypeptide to be expressed, and/or a selectable marker element. A preferred reporter gene incorporated into the vectors of the present invention is that of the human placental heat resistant secreted

alkaline phosphatase (SeAP) gene. Where the above elements are not already present in the vector to be used, they may be individually obtained and ligated into the vector. Methods used for obtaining these elements are well known to the artisan of ordinary skill in the art.

Especially preferred embodiments comprise the administration of synthetic DNA molecules encoding HIV *gag* and synthetic DNA molecules encoding modified forms of HIV gag. Another preferred embodiment of the present invention is the administration of DNA encoding influenza HA/georgia. Other preferred embodiments include the administration of DNA encoding proteins of the herpes simplex virus (HSV), such as gB and del gD, and the human papillomavirus (HPV).

Synthetic DNA molecules encoding HIV *gag* and synthetic DNA molecules encoding modified forms of HIV *gag* are described and claimed in co-pending patent application Serial Nos. 60/037846, 60/037854 and 09/017981, which are hereby incorporated by reference. Preferably, the codons of the synthetic molecules are designed so as to use the codons preferred by the projected host cell. The synthetic molecules may be used as a polynucleotide vaccine which provides effective immunoprophylaxis against HIV infection through neutralizing antibody and cell-mediated immunity.

Other polynucleotide formulations suitable for use in this invention are viral particles. Examples include adenovirus particle formulations, adeno-associated virus (AAV) particles, and alpha virus particles. One skilled in the art will recognize others which can also be advantageously introduced by needleless injection.

The amount of expressible DNA or polynucleotide to be introduced into a vaccine recipient will depend on the strength of the transcriptional and translational promoters used and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 ng to 100 mg, and preferably about 0.1 mg to 10 mg is administered directly into muscle tissue. It is also contemplated that booster vaccinations are to be provided at, for example, monthly or later time points. Following vaccination with HIV polynucleotide immunogen, boosting with HIV protein immunogens such as gp160, gp120, and *gag* gene products is also contemplated. Parenteral administration, such as intravenous, intramuscular, subcutaneous or other means of administration of interleukin-

12 protein, concurrently with or subsequent to parenteral introduction of the PNV of this invention may also be advantageous.

The polynucleotide or any DNA utilized in the methods of the present invention may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipients' immune system. Alternatively, the polynucleotide or DNA is complexed or viral form. Preferably the polynucleotide or other DNA is in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile buffered saline. Alternatively, the polynucleotide or DNA may be associated with liposomes, such as lecithin liposomes or cationic, anionic, or neutral lipids mixtures or other liposomes known in the art, such as a DNA-liposome mixture, or complex. Further, the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein, aluminum salts and/or other carrier. Agents which assist in the cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used to advantage. These agents are generally referred to herein as transfection facilitating reagents and pharmaceutically acceptable carriers.

Preferably, the polynucleotide used in the instant invention is administered intramuscularly, subcutaneously or both.

Another embodiment is a method of inducing prolonged gene expression through the injection of polynucleotide via the needleless injection system for gene therapy.

The following non-limiting Examples are presented to better illustrate the present invention.

EXAMPLE 1

Materials

A. Needleless Jet Injection Device

A BIOJECTOR TM2000 needleless injection device, a set of disposable syringe tips (Nos. 2, 3, 4, 5, and 7) and CO_2 cartridges were purchased from Bioject, Inc. (Portland, Oregon).

B. Animals

Animals were purchased from either Charles River, Covance or Jackson Laboratory and housed and treated following IACUC guidelines for the care and use of laboratory animals. Duncan Hartley guinea pigs of approximately 250 grams were purchased from Covance and allowed to acclimate to the animal facility for at least two weeks. New Zealand White rabbits were purchased from Covance at approximately 3kgs. and African green and rhesus monkeys were acquired from Charles River (Key Lois, FL and New Iberia Research Center, New Iberia, LA). The anesthesias Ketamine and rhompon were purchased from Phoenix Scientific (St.Joseph, MO).

C. DNA

Plasmid DNA was fermented and purified as described previously in the art. Either one of two transgenes were incorporated into the plasmids—influenza HA/georgia or HIV-1 gag driven by the CMV promoter—for use in the immunogenicity studies. V1Jns-HA (PR8) encoding the haemagglutinin (HA) from A/PR/8/34 was used in the influenza study. A plasmid containing the human placental heat resistant Secreted Alkaline Phosphatase (SeAP) reporter gene was constructed, also driven by the CMV promoter in the same vector backbone as the HA or gag constructs.

D. Assay Reagents

i. HA/georgia Haemagluttinin Inhibition: A stock solution of 2 mg/mL plasmid V1Jns-HA (PR8) encoding the haemagglutinin (HA) from A/PR/8/34 in 0.15 N saline was diluted with sterile 0.01 M phosphate-buffered saline (pH 7.4) to a final concentration of 10 μg/mL. DNA solutions were kept on ice until administered. Groups of four African green monkeys of either sex weighing between 3.8 and 7.1 kg were inoculated with plasmid intramuscularly in both quadriceps muscles. Each injection site received 0.5 mL of inoculum for a total dose of 10 μg of plasmid per animal. Animals receiving needle injections were injected in the frontal aspect of both thighs in the largest part of the quadriceps muscle near its midpoint. Animals receiving BIOJECTOR™ injections were injected using the No. 3 cartridge in the anterolateral aspect of each thigh. Injections were given at the beginning of the experiment, with booster immunizations being given 6 and 18 weeks later. Blood was collected at the beginning of the experiment and at 2-4 week intervals thereafter and was assayed for antibodies to the A/PR/8/34 virus by haemagglutination inhibition (HI) using chicken red blood cells with 4 HA

units of A/PR/8/34 virus, and by ELISA using whole formalin-inactivated A/PR/8/34 virus coated onto 96-well plates and developed with peroxidase-conjugated antibody to human IgG.

ii. Anti-gag Ab ELISA: Antibody ELISA assays were performed with Nunc immunosorb 96 well plates. HIV p24 antigen protein was purchased from Intracell. Secondary antibody conjugated with the Alkaline phosphatase was purchased from Jackson Immuno Research (West Grove, PA) for the guineas pigs. BSA, Tween-20 and OPD substrate was purchased from Sigma Chemical (St. Louis, MO).

iii. SEAP expression levels: PHOSPHA-LITE™ SeAP expression level kits were purchased from Tropix (#BP300, Bedford,MA)), and reagents were prepared according to the instructions in the kit. White 96-well luminometer plates were purchased from Fisher Scientific (#142-45-181).

E. Instruments

Optical densities of the ELISA dilutions were read on a Bio Tek Instruments UV900HDi 96 well plate reader. Luminescence from the serum SeAP levels was read on a Dynex luminometer. Gel negatives were scanned on a Bio Rad GS-700 Imaging Densitometer and stored on a PC.

EXAMPLE 2

In Vitro Characterization to Investigate and Determine the Integrity of the Polynucleotides After Needleless Jet Injection

A. Red Dye on Pig Skin

Physical characterization studies were conducted in order to evaluate the suitability of needleless injection on polynucleotide formulations. In a series of experiments designed to visualize and model the needleless jet injection into human tissue, aqueous phenol red dye solutions were injected into 20CC syringes with luer lock ends cutoff, and the syringe body filled with a 5% agarose gel. In order to visualize events upon injection through human skin, pig skin, purchased at a supermarket and was placed between the needleless syringe tip and an agarose filled syringe as a model system.

In photographs taken at the time, one can observe the BIOJECTOR™ aligned vertically above the syringe, pointed down and the red

dye in the syringe tip was clearly visible just before pulling the trigger on the needleless injection device. In a second image, one observed that the trajectory and profile of the Phenol red dye as it enters the agarose cylinder was quite narrow as it emerged from the syringe tip and slowly expanded trnasverse to the axis of propagation up to about three times its initial collimated diameter. If a section of pig skin was placed between the BIOJECTOR™ tip and the agarose cylinder, simulating what happens to the DNA as it passes through human skin, the profile of the jet is broadened immediately after it passed through the pig skin and it appeared turbulent to the eye, as if the orifice of the skin hole created a surface which generated vorticity. It appeared as if the injectate enters more broadly with pig skin than without. Without wishing to be bound by theory, it is concluded that this broadened jet facilitates in finding structural planes of little resistance in which the polynucleotide formulation is able to spread broadly.

B. Gel - after pass through at 100 mcg/ml

HA georgia DNA at 100 μg/ml was prepared in a stock solution. The DNA was passed through various syringe tips or syringe/needle combination, into a conical tube, agarose gel, or pig skin covered agarose gel (a model for human skin on muscle). Samples were loaded into either No. 3 or 7 syringe tips, injected, and collected. DNA was then run on a gel in triplicate and the quantity of supercoiled (S.C.) and open-circle (O.C.) DNA was determined relative to a standard curve. The relative values of super-coiled to total DNA (SC + OC) was determined for each sample.

Fig. 1 shows the results of the gel quantification of the component of supercoiled DNA (SC) to total DNA (SC + OC). The control sample (DNA solution befor other treatments) is indicated on the left with a relative value of 1.000. The 23g needle was statistically indistinguishable from the untreated control sample in the percentage of DNA in the supercoiled conformation. As a further control, incubating the DNA in a syringe tip did not degrade the DNA. When shooting the DNA from a No. 7 syringe tip into a conical tube (little to no frictional resistance in air), one recovered about 67% of the DNA in the original supercoiled state, whereas when the same tip was used but injected into a 5% agarose gel, only about 45% remained, thus 55% became nicked and reverted to the open circle or linear conformation. It is clearly noted that the additional passage through a layer of pig skin did not alter the

percentage of supercoiled DNA, although the error bars are larger reflecting a greater variability in the triplicate measurements. Finally, it should be noted that when the DNA was injected into the agarose from a No. 3 tip (with a smaller diameter orifice conferring a greater shear modulus and less force of penetration) rather than a No. 7 syringe tip (with a larger diameter orifice), the percentage of supercoiled fell from 45 % to nearly 32 %. Thus it appears as if at this low DNA concentration of 100 mcg/ml that the shear forces at the exit orifice of the syringe tip are responsible for the shear modulus on the DNA.

C. Gel - slow vs. fast at 2 different concentrations

Stock gag DNA at both 1 mg/ml and 5 mg/ml in normal saline was loaded into either a BIOJECTOR™ syringe tip No. 2 or No. 5. It was either then expelled either slowly in a dropwise fashion by pushing on the plunger slowly by hand into an eppendorf tube, or fired from the BIOJECTOR™ 2000 needleless jet injection device into a conical 50 ml disposable centrifuge tube, where it was collected and transferred to an eppendorf with a P1000 Gilson Pipetteman.

Samples were prepared as above and loaded onto a 1% agarose gel, run for 90 minutes at 70 V/cm, stained with 20 μg /ml Ethidium Bromide and destained for 30 min in deionized water on a rocking stage.

Gels were placed on a photodocumentation setup and photographed with Polaroid film which produces both a positive and negative photoemulsion. The negative was washed in water for at least several hours before being exposed under UV light for 10-30 seconds. Development occurred as per the film manufacturer's instructions.

The gel negative optical density was scanned into the computer on a Bio-Rad GS-700 Imaging Densitometer with the resolution set at 42 μm and the quantitation was done using Molecular Analyst Software, Version 1.3 (Bio Rad Lab) to analyze the scanned data.

As shown in Fig. 2 , a number of samples were passed through either a No. 2 (small diameter orifice) or No. 5 (large diameter orifice) BIOJECTOR™ syringe tip, either slowly by hand in a drop wise fashion or expelled by the burst of CO_2 gas from the BIOJECTOR™. Two sets of samples were run, 1 mg/ml (those on the left of the figure) and 5 mg/ml (on the right). Lane position is enumerated from left to right sequentially across the gel. In the left-most lane of each set (lanes 1 and 6), a control was run in which no

manipulation took place. In lanes 2 and 3, DNA was loaded into a No. 2 syringe tip and expelled, either slowly by hand or fast via the CO_2 expulsion from BIOJECTOR™. Although there appears no gross difference in the gel pattern, a slight "tail" is discernible running ahead of the supercoiled band. In lanes 4 and 5, the same was done with a No. 5 syringe tip. There, the slow sample (by hand) appears identical to that of the control whereas the BIOJECTOR™ expelled sample shows a clear smear tail running ahead of the supercoiled band.

Looking at the identical experiments done with 5 mg/ml DNA in the right set of lanes, one notices "tails" in lanes 8 and 10, but these are less pronounced at the more viscous higher DNA concentration. Thus it appears that a more viscous and/or higher DNA concentration protects DNA from shear forces. Moreover, at the more viscous higher concentrations, greater shear forces are occurring in the syringe tips with the larger diameter (the No. 5).

D. Gel - slow vs. fast at high concentration

Gels were carried out with HIV-gag DNA, at 5.5 mg/ml in saline, each vial containing 0.8 ml DNA solution.

The effects of the BIOJECTOR™ syringe on the supercoiled content of the DNA was determined by agarose gel electrophoresis of each of the ejected DNA samples. As a control, the starting plasmid DNA was also applied to the same gel. An additional control included a sample of HIV-gag DNA that was ejected through a 28 gauge needle. Aliquots of each DNA sample, containing 18 ng of DNA, were applied to separate lanes of a 1% agarose gel. Following electrophoresis, the gel was stained with ethidium bromide and photographed under UV light. A negative of the gel photograph was then scanned using a Bio-Rad GS-700 densitometer. The quantity of DNA in each band on the gel was determined using a Molecular Analyst (ver. 1.3) software program (Bio-Rad), based on the band intensities produced by known amounts of supercoiled (SC), open-circular (OC) and linear DNA standards applied to the same gel. To ensure accurate results a standard curve was generated using the band intensities of the DNA standards and the mass of DNA applied to each lane of the gel for each form of DNA (SC, OC and linear).

A quadratic equation was fitted to the data for each of the standard curves (SC, OC and linear). The correlation coefficients of all standard curves were >0.99.

To determine the effects of ejecting plasmid DNA through a BIOJECTOR™ syringe on the supercoiled content of the DNA, supercoiled plasmid DNA at 5.5 mg/mL in saline was ejected through either a No. 2 or a No. 5 BIOJECTOR™ syringe tip filled with either 200 mcL (No. 2 syringe tip) or 500 mcL of HIV-gag DNA (No. 5 syringe tip). The effects of the BIOJECTOR™ syringe on the supercoiled content of the DNA was determined by agarose gel electrophoresis.

Agarose gel electrophoresis of plasmid DNA ejected through a Biojector syringe or a 28 gauge needle and quantitation are shown in Figure 3. The positions to which SC, OC and linear HIV-gag DNA migrate on the gel are indicated on the left side of the gel. Lane 1 shows the starting DNA control. Lane 2 shows the DNA after being ejected through a 28 gauge needle. Lanes 3 and 4 show DNA after ejection through the No. 2 Biojector syringe slowly by hand (lane 3) or with the Biojector device (lane 4). Lanes 5 and 6 show the DNA after ejection through the No. 5 Biojector syringe slowly by hand (lane 5) or with the Biojector device (lane 6).

The starting material (lane 1) was mostly SC DNA, with only a small quantity of OC DNA observed. Quantitation of the bands in lane 1 indicated that the starting plasmid DNA was 91% SC, 9% OC, 0% linear. The results shown in lanes 2, 3 and 5 indicate that ejection of HIV-gag DNA through a 28 gauge needle or slowly through either a No. 2 or a No. 5 BIOJECTOR™ syringe did not have a significant effect on the percentage of SC DNA and caused no detectable loss of material. However, in lanes 4 and 6 small amounts of linear DNA were observed in the gel, presumably due to shearing of the DNA caused by fast ejection through the BIOJECTOR™ syringe. Quantitation of the DNA bands in lane 4 indicated that the DNA was 93.32% SC, 5.91% OC and 0.78% linear. These results suggest that a small amount of linear DNA was produced by the conversion of OC DNA to linear. Quantitation of the DNA bands in lane 6 indicated that the DNA was 93.08%

SC, 5.70% OC and 1.22% linear. These results also indicate a small amount of conversion of the OC DNA to linear, during the fast ejection through the BIOJECTOR™ syringe. Quantitation of the DNA bands in all lanes indicated complete recovery of the DNA through the needle and BIOJECTOR™ syringes. In summary, ejection of plasmid DNA through a No. 2 or No. 5 BIOJECTOR™ syringe caused no loss of SC DNA and only a small conversion of OC to linear DNA. Since the starting DNA contains only a small amount of OC DNA, the amount of linear DNA generated was near 1% of the total DNA and should have no detectable effect on the biological potency of the ejected material.

E. Effect of shear forces on the stability of plasmid DNA

Using a Carri-Med Rheometer, fitted with a cone and plate, three concentrations of DNA plasmid in either saline of saline/glycerol were subjected to various shear forces. The shear force applied to the DNA in these samples was the maximum that could be applied with this Rheometer and the best one could do to approximate the needleless injection device. Plasmid DNA was tested at 25, 250 and 2500 mcg/mL in saline. Only the 25 and 250 mcg/mL concentrations were tested in the saline/glycerol solution. The reasoning behind the addition of glycerol was to increase the viscosity to that of the bulk DNA (2.5 mg/mL) to prevent shearing of the low dose concentration of DNA. Shear forces were applied to the samples at 10 and 25°C, with and without glycerol. For the control samples the plate (containing sample) was raised to the cone but not rotated; therefore no shear force was applied.

The results are presented in Fig. 9. To determine if the samples had been sheared the conversion of supercoiled DNA to open-circular DNA was determined by agarose gel electrophoresis. In summary, the data indicates that some shearing of the DNA occurred in the low concentration (25 mcg/mL) DNA plasmid sample when diluted in saline and the shear force applied at 25°C. However, the same sample in glycerol/ saline was not sheared. These results suggest that there may be a viscosity effect to the shearing of DNA. Moreover, since the 25 mcg/mL appears to be more susceptible to shearing than the samples having higher DNA concentrations, there is most likely a concentration effect as well. This data thus support the

notion that higher DNA concentrations, and thus higher viscosities, protect the DNA when injected from a needleless injection device.

EXAMPLE 3

Bio-Distribution Syringe Tip Selection Studies To Determine The Proper Syringe Tip For The Skin/Fascia/Muscle Composition Of A Given Species And Injection Site.

Pilot experiments were conducted using Trypan Blue Stain 0.4% (Gibco # 15250-061) in order to visualize the extent of bio-distribution within the target tissue. The diluent, phosphate buffer saline, pH 7.2 (PBS) was ordered from Merck Research Laboratory's internal laboratory supply group. Photographs were taken with a Nikon N70 35mm camera using Kodak elite II 400 slide film.

A. Guinea Pig Pilot Experiments

Stock trypan blue was mixed either 1:1 with PBS, 200 μg/ml or 2 mg/ml gag DNA in saline, or in a 77 % ratio of DNA to trypan blue in order to achieve a final concentration of 5 mg/ml gag DNA with trypan blue. DNA/trypan blue was loaded into the weakest penetrating syringe tip, a No. 2, to 200 μl following the manufacturer's instructions, tapping hard to get rid of air bubbles that may form at the interface of the plunger surface and the o-ring seal. A fresh or well charged CO_2 was previously loaded into the BIOJECTOR™ device, the syringe tip was locked into place.

The guinea pigs were anesthesized with a mixture of ketamine (44 mg/kg) and xylazine (5 mg/kg) and shaved of their hair in the area of the upper hamstring. Then an injection of 200 μl injectate was given to each guinea pig in the fleshy part of the upper hamstring, using a flat plastic ruler underneath the muscle tissue to support it when making the injection. The exact site of injection was determined from the anatomy of each animal. The bone that runs from the knee towards the spine was palpated. At a point two thirds the way from the knee to the spine and 4-6 mm posterior, a dot was made with a magic marker in order to help line up the BIOJECTOR™ syringe tip. As per the manufacturer's instructions, firm pressure was absolutely necessary to insure the skin retention ring of the BIOJECTOR™

syringe tip made good contact resulting in a good deep intra-muscular injection; otherwise, a loosely pressed syringe tip would leak injectate out the sides of the syringe rather than into the skin and into the muscle.

After injection of the dye, often at different DNA concentrations and hence different viscosities of the injectate, the guinea pigs were sacrificed and photographs of the closed skin, open skin, cross section of the muscle, and pool of injectate were taken.

In a separate experiment, it was attempted to immunize the guinea pigs awake without anesthesia. This necessitated a larger set of hands in order to restrain the guinea pig on the table. In general, we found it advantageous to support the guinea pig on its side on top of 1-2 inch block of wood, roughly 6 inches wide by 10 inches long. This allowed the guinea pig to lay just above the surface of the bench, permitting better access to restrain the animal with gloved hands. Also, it was found better to soothe the animal to cover their eyes while being restrained.

After injections were made with a No. 2 tip at various concentrations of DNA, it was decided to move to the next higher syringe number, in this case a No. 3 tip, until examination of the muscle tissue either demonstrated a good intramuscular injection or one which was too strong and penetrated too far. From this procedure, the appropriate tip was selected for the guinea pig upper hamstring muscle group. A direct comparison to a 27g1/2 insulin syringe/needle as a control was also made.

After conducting several pilot experiments with the BIOJECTOR™ 2000 on guinea pigs, we arrived at the conclusion that the No. 2 syringe tip was the only option to be used on the upper fleshy portion of the hindleg hamstring of the guinea pig. Moreover, it was discovered that to get consistent and reproducible injections into the muscle that it was necessary to support the guinea pig hind limb with a plastic ruler in order that there be direct support for the tissue below the BIOJECTOR™ syringe tip.

An injection of trypan blue/DNA using a 27g1/2 insulin syringe/needle resulted in a bolus of blue DNA forming in the upper hamstring muscle mass. When DNA/trypan blue was injected using a No. 2 syringe tip intramuscularly to the guinea pig, it was observed that the blue DNA solution extended over a much larger area and volume of muscle tissue. Structural planes in the tissue, for instance at fascia or layers of muscle fibers,

enabled the blue DNA solution to spread transverse to the axis of injection, over several centimeters in distance from the injection axis, often in more than one structural plane. The interpretation is thus that because the resistance to flow or impeadance can be reduced at a structural interface within the tissue, this enables the formulation to spread further and bathe a larger surface area and volume of tissue than a "bolus" can from a sryinge/needle combination. Moreover, the No. 3 syringe tip nearly exited the opposite side of the hindlimb, clearly indicating too powerful of a syringe tip for use with the guinea pig hamstring. Thus, it was determined that only the No. 2 tip was workable for use with guinea pigs.

Initial experiments were done with animals under anesthesia until we were able to demonstrate no adverse effects with the BIOJECTOR™ needleless jet injection device. Then, we restrained the guinea pigs by hand on a wood block and proceeded to immunize them without anesthesia An alternate site was identified, lower on the hamstring near the back of the knee joint; however, there is considerably less muscle tissue just below the skin surface and so it would be preferable for BIOJECTOR™ injections to use the upper hamstring.

The bio-distribution pattern observed after the DNA was injected with the BIOJECTOR™ was much broader than that of a 27g1/2 insulin syringe. While not intending to be bound by theory, it is believed important to bathe a larger surface area of muscle and other tissue with DNA in order to get a larger, more pronounced immune response. It is believed that as the jet of DNA penetrates the tissue, it finds paths of least resistance at structural planes such as fascia or interfaces between muscle groups, and is able to travel transverse or perpendicular to the axis of injection. This is unique to jet injection since the solid wall of a standard metal needle precludes anything but a bolus of DNA from forming at the end of the needle.

B. Rhesus Pilot Experiments

As in the guinea pig bio-distribution syringe tip selection experiments, various BIOJECTOR™ syringe tips were loaded with DNA/trypan blue combinations at various concentrations and injected into various muscle groups of a rhesus monkey that had previously been scheduled for euthanasia for other compelling ethical considerations. The rhesus was anesthesized with a dose of ketamine (10 mg/kg) . Of primary interest was the

intra-muscular injection into the deltoid or quadricep muscle (proximal and distal). Other muscle groups injected included the bicep, tricep, hamstring (proximal and distal), and calf on both sides of the animal.

After injection of 0.5 ml per site with DNA/trypan blue, the animal was euthanized with Euthanasia solution. Immediately thereafter, the same photodocumentation regimen was conducted as outlined above in the guinea pig studies. Syringe tips ranged from No.2 to No. 7 with DNA concentrations from zero to 5 mg/ml.

After expiration, the various sites were opened and documented on film. Injections of 5 mg/ml DNA with a No. 3 syringe tip into a rhesus deltoid produced a well dispersed pattern of blue dye indicating that the DNA has moved laterally away from the injection site. As done earlier with guinea pigs, we verified that one syringe tip size smaller did not penetrate well into the muscle tissue of choice (only penetrated a few millimeters into the muscle mass if at all) or that one size larger penetrated too deeply, either cutting the muscle tissue, scattering after striking bone, or running through to the other side.

When examining injections on the rhesus, it was noted that the skin was thinner on the lower extremities of the monkey and therefore less resistant to the needleless injection, thus emphasizing the importance of proper syringe tip seletion for a given skin/muscle site on a given species of animal.

C. Rabbit Pilot Experiments

Bio-distribution syringe tip selection experiments were conducted on awake rabbits in their sacrospinalis back muscle. The site of injection for the anterior sites (left and right) are given approximately 2-3 cm below the level of the posterior rib cage. The posterior sites (left and right) are given approximately 2-3 cm above the iliac crest. The rabbits were shaved earlier and injections were made with syringe tips ranging from No. 2 to No. 7 at DNA concentrations from 0 (PBS) to 5 mg/ml HIV gag DNA. Differently than before, after injection, each side of the sacrospinalis muscle was dissected out and then thinly sliced to visualize the extent of the DNA/ trypan blue dye distribution. Rabbit skin, even after it was shaved, was much thicker and resistant to needleless jet injection. It was necessary to use a No. 5 syringe tip when using highly viscous 5.5 mg/ml DNA as vaccine to get a deep 1-2 cm

injection. As before, one size less powerful, the No. 4 syringe tip, produced an insufficient injection (penetrating up to 5 mm into the muscle), whereas one size more powerful, the No. 7 syringe tip, produced a jet track that actually cut through the muscle.

EXAMPLE 4

Immunogenicity Studies

A. African Green HA/Georgia Immunogenicity Experiment

Ketamine was used (10 mg/kg) to put the animals asleep. A stock solution of 2 mg/mL plasmid V1Jns-HA (PR8) encoding the haemagglutinin (HA) from A/PR/8/34 in 0.15 N saline was diluted with sterile 0.01 M phosphate-buffered saline (pH 7.4) to a final concentration of 10 μg/mL. DNA solutions were kept on ice until administered. Groups of four African green monkeys of either sex weighing between 3.8 and 7.1 kg were inoculated with plasmid intramuscularly in both quadriceps muscles. Each injection site received 0.5 mL of inoculum for a total dose of 10 μg of plasmid per animal. Animals receiving needle injections were injected in the frontal aspect of both thighs in the largest part of the quadriceps muscle near its midpoint. Animals receiving BIOJECTOR™ injections were injected using the No. 3 syringe tip in the anterolateral aspect of each thigh. Injections were given at the beginning of the experiment, with booster immunizations being given 6 and 18 weeks later. Blood was collected at the beginning of the experiment and at 2-4 week intervals thereafter and was assayed for antibodies to the A/PR/8/34 virus by haemagglutination inhibition (HI) using chicken red blood cells with 4 HA units of A/PR/8/34 virus, and by ELISA using whole formalin-inactivated A/PR/8/34 virus coated onto 96-well plates and developed with peroxidase-conjugated antibody to human IgG.

As shown in the Figures 4(A) and 4(B), three needle injections of 10 μg of DNA did not elicit a significant antibody response by HI or ELISA. However 3/4 monkeys given 10 μg of DNA using the BIOJECTOR™ responded with antibodies detectable by both HI and ELISA after the third dose.

B. Guinea Pig HIV Gag DNA Dose Response Experiment

Cohorts of 6 guinea pigs were anesthetized with ketamine/rompun combination. At 0, 4, and 8 weeks, cohorts were immunized intramuscularly with 200 µl of V1Jns-HIV gag DNA vaccine per upper hamstring via either a No. 2 syringe tip injection with the BIOJECTOR™ or a 27g1/2 insulin syringe/needle combination. Animals received either a 200 µg/ml, 1 mg/ml or 5 mg/ml concentration of HIV-1 gag DNA (dose = 80 mcg, 400 mcg, or 2 mg, respectively).

At 4, 8 and 13 weeks, animals were bled and the sera collected and stored at 4°C until assayed. Anti-gag Ab ELISAs were measured from sera and titers assigned from the OD plate readings by endpoint titer (criteria of greater than two fold above background for a sero conversion). Typically first dilutions were 100:1 but were sometimes as high as 400:1, with serial dilutions down the plate of typically 3X or 4X. Endpoint titers were assigned where optical densities of the chromogenic agent (OPD) were greater than 2.5 times above the background level of a well. Typically, backgrounds levels on the plate were around 0.040 OD, so end-point titer thresholds were typically selected at 0.100 OD.

As shown in Fig. 5(A) guinea pigs immunized with the BIOJECTOR™ revealed enhanced anti HIV-1 gag antibody titers, as measured by ELISA, 4 weeks post a single dose of either 80, 400 or 2000 mcg of HIV-1 gag DNA. At the lowest dose, only one of six animals sero-converted after injection with a needle, whereas four of 5 animals assayed, sero-converted after BIOJECTOR™ immunization. At the intermediate dose, only 2/6 animals seroconverted via needle injection whereas 6/6 seroconverted via needleless jet injection. Moreover, the cohort geometric mean titer (GMT) exhibited a ten fold enhancement at the 400 mcg dose. At the highest 2 mg dose, only 2/6 animals seroconverted via needle immunization whereas 6/6 seroconverted via BIOJECTOR™ needleless injection. Surprisingly and unexpectedly, the cohort GMTs differed by a significant 80 fold enhancement.

Fig. 5(B) shows the Anti-gag antibody responses from the serology four weeks later after a second immunization with the identical vaccines. All BIOJECTOR™ immunized cohorts in this experiment have 100% seroconversion, whereas the needle injected cohorts do not. At the low dose, the BIOJECTOR™ conferred a 12 fold enhancement in Ab titer, while at the intermediate dose it conferred a 144 fold enhancement. This unusually high

enhancement is slightly exaggerated in that it appears as if three guinea pigs did not respond at all after needle immunization at the intermediate dose, thus depressing the needle GMT and thus boosting the relative enhancement factor. Regardeless, at the high dose, there is a 38-fold enhancement from needle to needleless syringe.

Finally, it should be noted that the variability in the animal response to the vaccine appears unexpectedly reduced after BIOJECTOR™ needleless jet injection compared to that observed with an insulin syringe/needle. In this experiment, all three of the BIOJECTOR™ injected cohorts are spread over 3 wells of dilution on the microtiter plate, whereas the syringe/needle groups are typically spread over four to five wells on a given plate.

C. Guinea Pig HIV Gag DNA Anesthesia/Site Experiment:

In a separate experiment, 8 cohorts of 5 guinea pigs each were immunized intramuscularly at 0 and 4 weeks with 200 µl of vaccine, again via either a No. 2 syringe tip injection with the BIOJECTOR™ or a 27g1/2 insulin syringe/needle combination. Moreover, animals received injections in either the upper hamstring where a large muscle mass was located or in the lower hamstring near the knee joint where traditional needle immunizations had been done previously in our group. In an effort to test the effect of anesthesia, half the animals were injected awake and half under anesthesia as indicated above. All guinea pigs received a 1 mg/ml solution of HIV-1 gag DNA in PBS (dose = 400 mcg).

At 3 and 7 weeks, animals were bled and the sera collected and stored at 4°C until assayed. Anti-gag Ab ELISAs were measured from sera and titers assigned from the OD plate readings by endpoint titer (criteria of more than two foldabove background for a sero conversion). Typically first dilutions were 100:1 but were sometimes as high as 400:1, with serial dilutions down the plate of typically 3X or 4X. Endpoint titers were assigned where optical densities of the chromogenic agent (OPD) were greater than 2.5 times above the background level a well. Typically, backgrounds levels on the plate were around 0.040 OD so end-point titer thresholds were typically selected at 0.100 OD.

A shown in Fig. 6, the data show no marked difference either with or without anesthesia. When comparing needle to BIOJECTOR™,

enhancements range from 3 fold to 8 fold. As before, BIOJECTOR™ cohorts exhibit improved rates of seroconversion and less animal to animal variability compared to syringe/needle, although with N=5 guinea pigs per cohort, the results are less persuasive. It should be noted that this serology is 3 weeks post dose 1, a week sooner than the results reported in Fig. 5A.

D. Rhesus HIV Gag DNA Immunogenicity Experiment

An immunogenicity study was initiated recently to look at immune responses, both humoral and cellular, in non-human primates all with HIV-1 gag DNA. Six cohorts of 3 rhesus monkeys each were immunized according to the following study protocol. Each animal received bilaterally 0.5 ml of vaccine in the deltoid (for a total volume of 1.0 ml), either by BIOJECTOR™ with No. 3 syringe tip or with a 25g5/8 needle. A 10 mg/kg dose of Ketamine was used as anesthesia.

Cohort 1 received a total dose of 5 mg of DNA via 25g5/8 needle while under anesthesia.

Cohort 2 received a total dose of 5 mg of DNA via BIOJECTOR™ with No. 3 syringe tip while under anesthesia.

Cohort 3 received a total dose of 5 mg of an HIV-1 gag DNA construct that lacked a leader sequence (DNA #80) via BIOJECTOR™ with No. 3 syringe tip while under anesthesia.

Cohort 4 received a total dose of 1 mg of DNA via 25g5/8 needle while under anesthesia.

Cohort 5 received a total dose of 1 mg of DNA via BIOJECTOR™ with No. 3 syringe tip while under anesthesia.

Cohort 6 received a total dose of 1 mg of DNA via BIOJECTOR™ with No. 3 syringe tip while awake, temporarily restrained by a collar during chair training.

Animals should be bled every other week. Sera will be assayed for Anti-gag Ab via ELISA as well as the cell mediated response characterized by Limiting Dilution Analysis of a CTL based assay.

EXAMPLE 5

Gene Expression Studies

A. Guinea Pigs Injected With The Heat Resistant Human Placental Secreted Alkaline Phosphatase (SeAP) Gene.

4 cohorts of 8 guineas pigs each were injected with 200 µl doses of a plasmid encoding the heat resistant human placental secreted Alkaline Phosphatase (SeAP) gene once, either with a 27g1/2 needle or BIOJECTOR™ needleless jet injection device with No. 2 syringe tip, both with and without anesthesia. Small quantities of blood were collected periodically over the first 2 weeks, then at weeks, 3, 4, and 8. Blood was spun and the sera removed for storage at -20°C until assayed.

SeAP experssion levels detected in the blood were determined following the manufactuer's instructions. Standard curves of Alkaline phosphatase in pooled guinea pig serum were used to quantitativly determine the quantity of active SeAP protein circulating in the guinea pigs.

In Fig.7, results are presented of the reporter gene activity of SeAP which was measured in guinea pigs injected once with either a high or low dose of SeAP encoding plasmid DNA, either by insulin syringe or BIOJECTOR™. The cohort averages are plotted as a function of time. The SeAP levels in both needle cohorts started low and increased over the first week as indicated by the solid circle and square. On the other hand, the cohort injected via BIOJECTOR™ did not demonstrate a large response (solid diamond) whereas the high dose BIOJECTOR™ cohort exhibited a 3 fold enhancement in SeAP activity at day 2, but steadily falls off during the first week. This dramatically different kinetics of gene expression between needleless and syringe/needle injection was not anticipated nor disclosed in the prior art and resulted in a surprising discovery. Moreover, the ability to take advantage of the apparent rapid increase in gene expression at short time scale may prove useful in certain other gene delivery and gene therapy applications, such as the delivery of hormones, receptor proteins, and ligands.

While not wishing to be bound by theory, it appears that there may be a synchronization between the faster kinetics of gene or antigen expression, coupled with an inflamatory or immune response at short times that leads to the large enhancement observed in anti-gag Ab end titers using ELISA.

EXAMPLE 6

Sizes of needleless injector tips are taken from US Patent 5,520,639,which is hereby inorporated by reference.ect, Inc.

syringe tip	nozzle diameter (inches)	nozzle diameter (mm)
No. 2	0.004	0.100
No. 3	0.006	0.150
No. 4	0.008	0.200
No. 5	0.010	0.250
No. 7	0.014	0.350

WHAT IS CLAIMED IS:

1. An improved method of inducing an immune response to a polynucleotide vaccine in an animal using a needleless injector comprising:

introducing the vaccine intramuscularly wherein the polynucleotide vaccine, upon entry into the animal, is not present in a bolus, and wherein the polynucleotide vaccine is substantially evenly distributed in an area of muscle tissue.

2. A method according to Claim 1 wherein the polynucleotide vaccine is a DNA vaccine.

3. A method according to Claim 2 wherein the polynucleotide vaccine is an HIV vaccine.

4. A method according to Claim 3 wherein the polynucleotide vaccine encodes for HIV *gag*.

5. A method according to Claim 2 wherein the polynucleotide vaccine encodes for Influenza HA.

6. A method according to Claim 1 wherein the introduction of the vaccine is controlled by passing the vaccine through an orifice having a predetermined diameter.

7. A method according to Claim 1 wherein the concentration of the polynucleotide vaccine is at least 5 μg/ml.

8. An method of delivering DNA to an animal for purposes of gene therapy using a needleless injector comprising introducing the vaccine intramuscularly wherein the polynucleotide vaccine, upon entry into the animal, is not present in a bolus, and wherein the polynucleotide vaccine, upon entry into the animal, is substantially evenly distributed in an area of muscle tissue.

9. A method according to Claim 8 wherein the polynucleotide vaccine is in an amount sufficient to effect the desired prophylactic or therapeutic response.

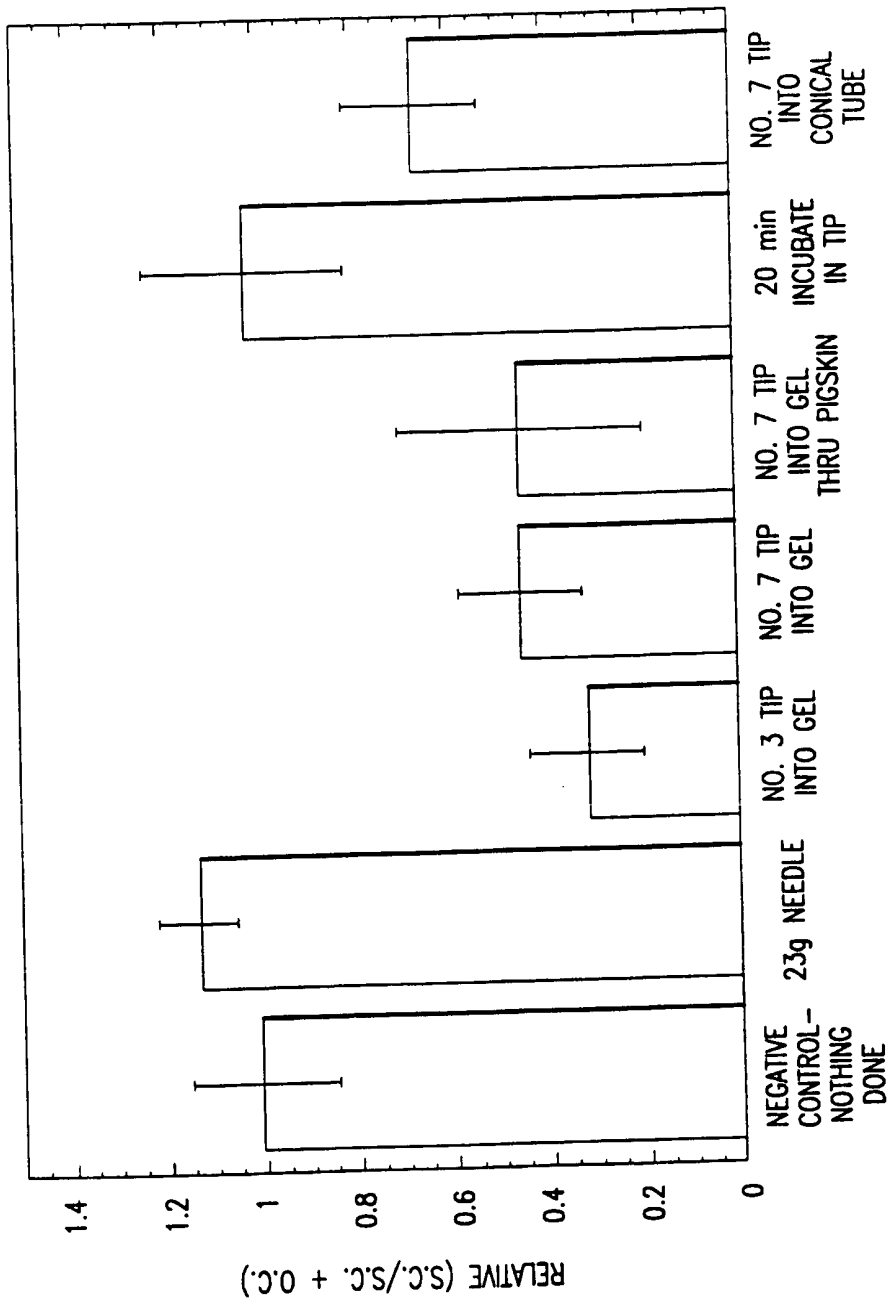

RELATIVE (S.C./S.C. + 0.C.)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
NEGATIVE CONTROL– NOTHING DONE
23g NEEDLE
NO. 3 TIP INTO GEL
NO. 7 TIP INTO GEL
NO. 7 TIP INTO GEL THRU PIGSKIN
20 min INCUBATE IN TIP
NO. 7 TIP INTO CONICAL TUBE
SAMPLE

FIG. 1

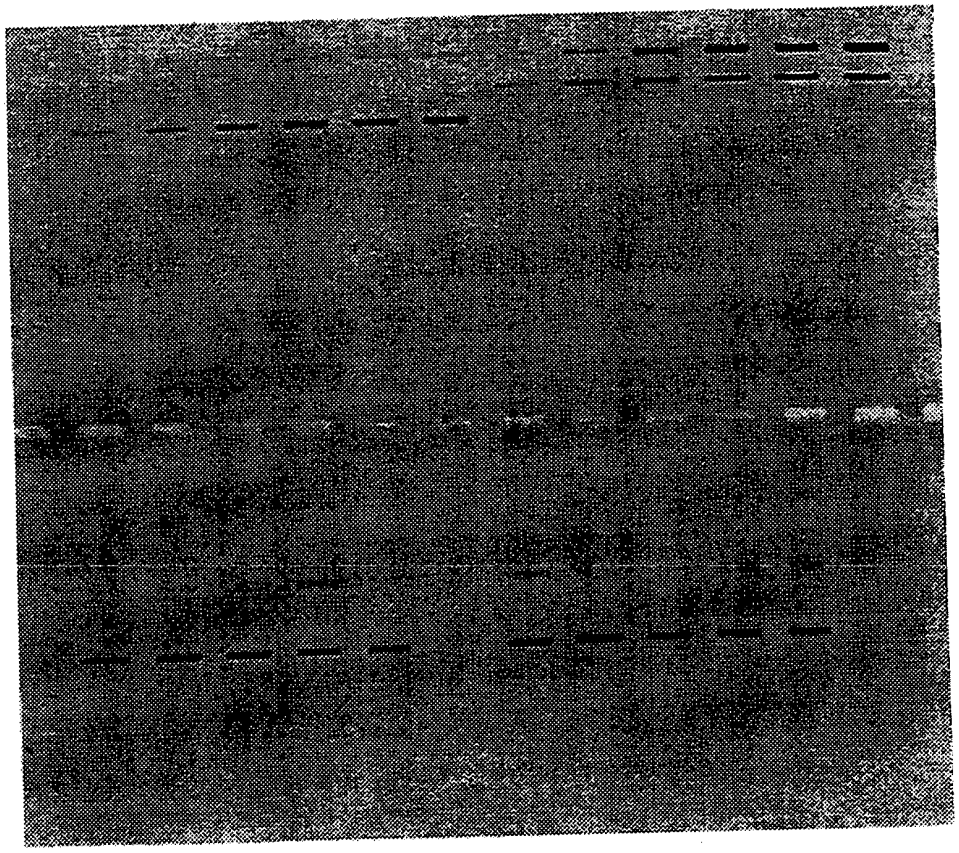
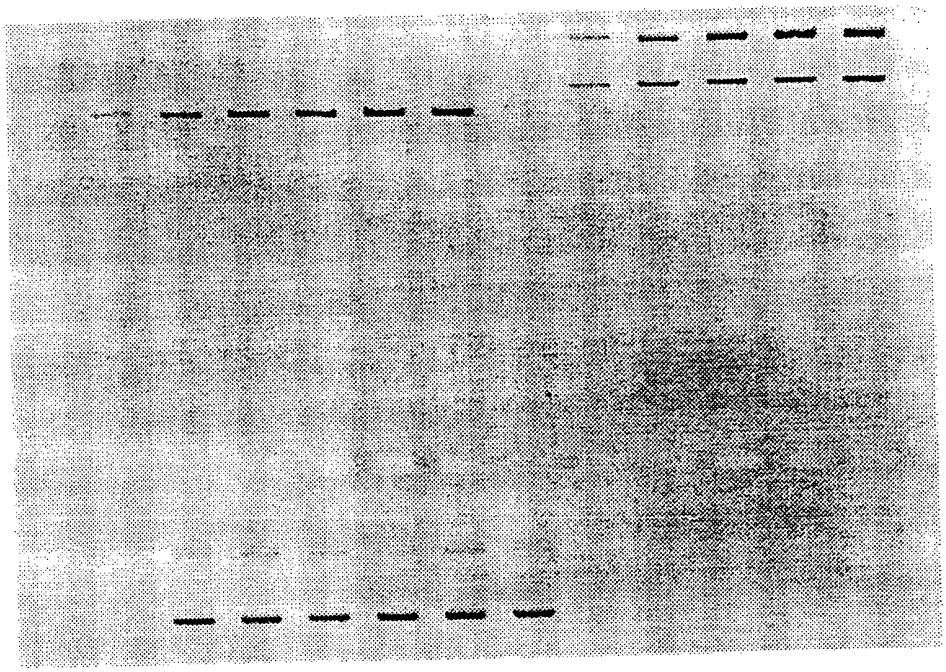


FIG.2A

lane	sample	Total DNA	%SC	%OC	%Linder
1	1 mg/ml control	17.16	94.41	5.59	0.00
2	1 mg/ml No.2 slow	17.68	94.97	5.03	0.00
3	1 mg/ml No.2 fast	14.49	97.59	2.41	0.00
4	1 mg/ml No.5 slow	17.20	93.03	6.97	0.00
5	1 mg/ml No.5 fast	12.08	97.22	2.78	0.00
6	5 mg/ml control	15.44	94.97	5.03	0.00
7	5 mg/ml No.2 slow	15.85	93.10	6.90	0.00
8	5 mg/ml no.2 fast	13.32	96.56	3.44	0.00
9	5 mg/ml No.5 slow	15.96	94.05	5.95	0.00
10	5 mg/ml No.5 fast	15.21	94.54	5.46	0.00

FIG.2B



1 2 3 4 5 6

FIG.3A

lane	Sample	Total DNA	%SC	%OC	%Linear
1	DNA control	14.14	91.24	8.76	0.00
2	needle	15.47	91.74	8.26	0.00
3	200 mcl No.2 slow	15.29	91.90	8.10	0.00
4	200 mcl No.2 fast	14.90	93.32	5.91	0.78
5	500 mcl No.5 slow	14.84	90.95	9.05	0.00
6	500 mcl No.5 fast	15.14	93.08	5.70	1.22

FIG.3B

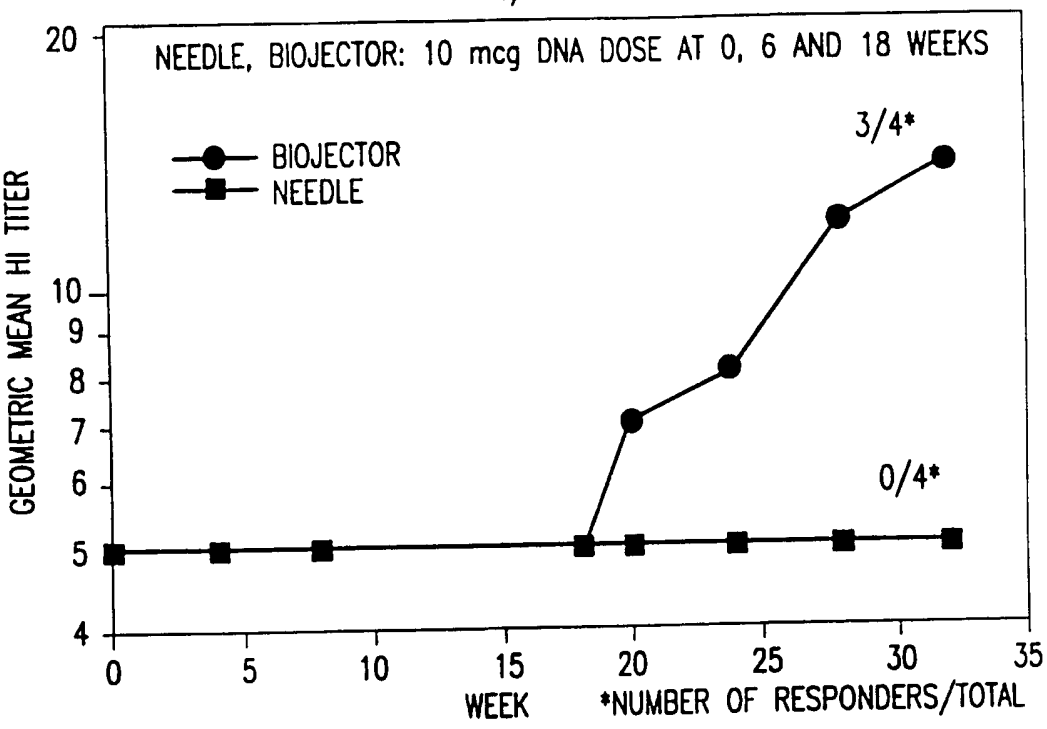

NEEDLE, BIOJECTOR: 10 mcg DNA DOSE AT 0, 6 AND 18 WEEKS
BIOJECTOR
NEEDLE
3/4*
0/4*
GEOMETRIC MEAN HI TITER
20
10
9
8
7
6
5
4
0
5
10
15
20
25
30
35
WEEK
*NUMBER OF RESPONDERS/TOTAL

FIG.4A

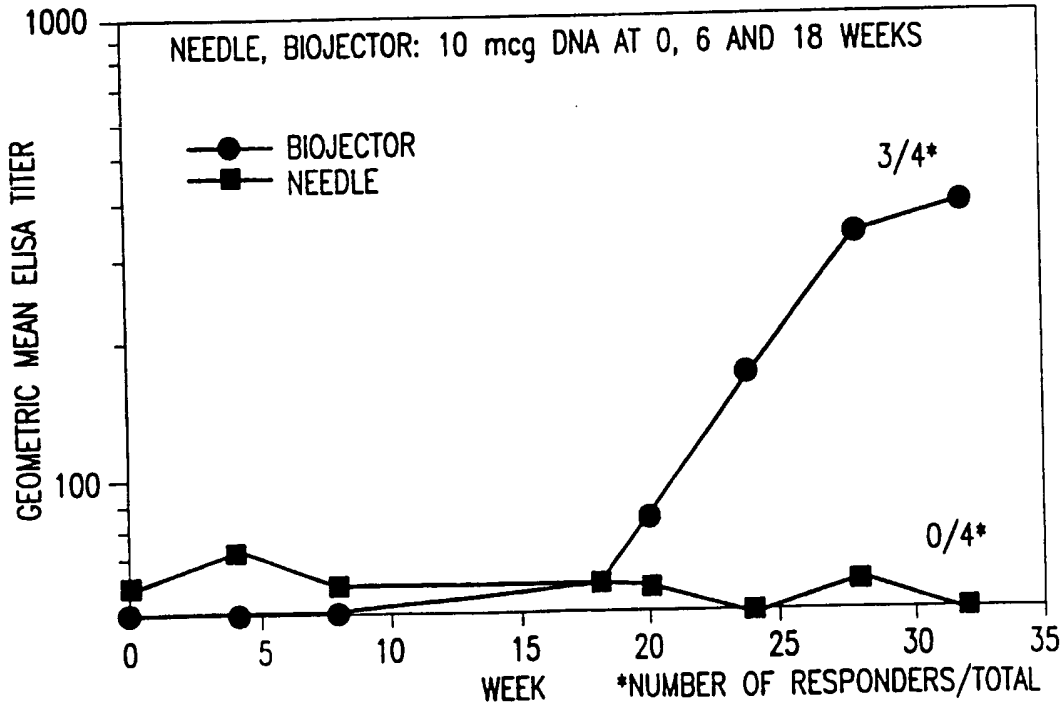

NEEDLE, BIOJECTOR: 10 mcg DNA AT 0, 6 AND 18 WEEKS
BIOJECTOR
NEEDLE
3/4*
0/4*
GEOMETRIC MEAN ELISA TITER
1000
100
0
5
10
15
20
25
30
35
WEEK
*NUMBER OF RESPONDERS/TOTAL

FIG.4B

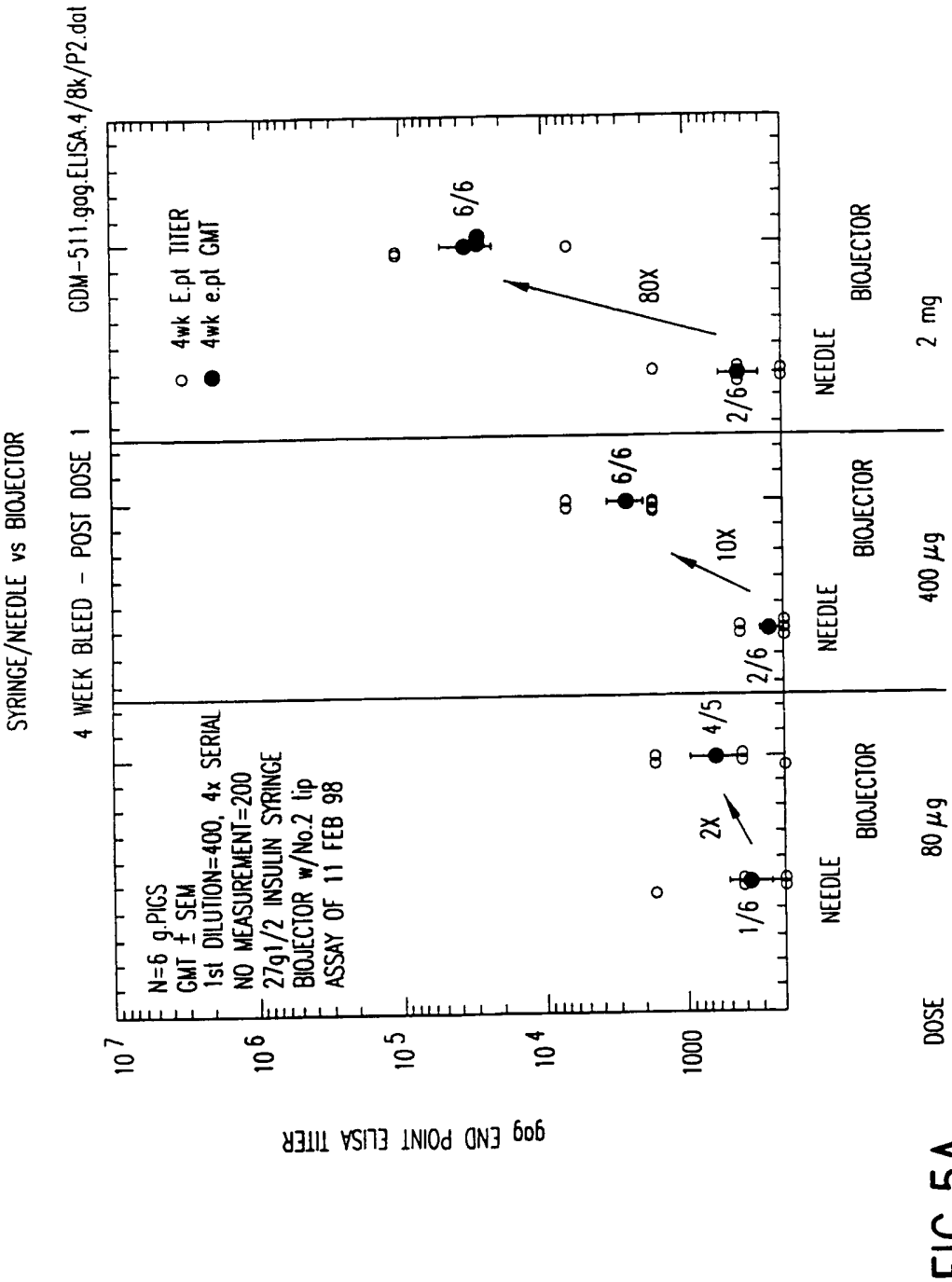

SYRINGE/NEEDLE vs BIOJECTOR
4 WEEK BLEED - POST DOSE 1
GDM-511.gog.ELISA.4/8k/P2.dol
4wk E.pl TITER
4wk e.pl GMT
N=6 g.PIGS
GMT ± SEM
1st DILUTION=400, 4x SERIAL
NO MEASUREMENT=200
27g1/2 INSULIN SYRINGE
BIOJECTOR w/No.2 lip
ASSAY OF 11 FEB 98
gog END POINT ELISA TITER
10^7
10^6
10^5
10^4
1000
1/6
2X
4/5
2/6
10X
6/6
2/6
80X
6/6
NEEDLE
BIOJECTOR
NEEDLE
BIOJECTOR
NEEDLE
BIOJECTOR
DOSE
80 μg
400 μg
2 mg

FIG.5A

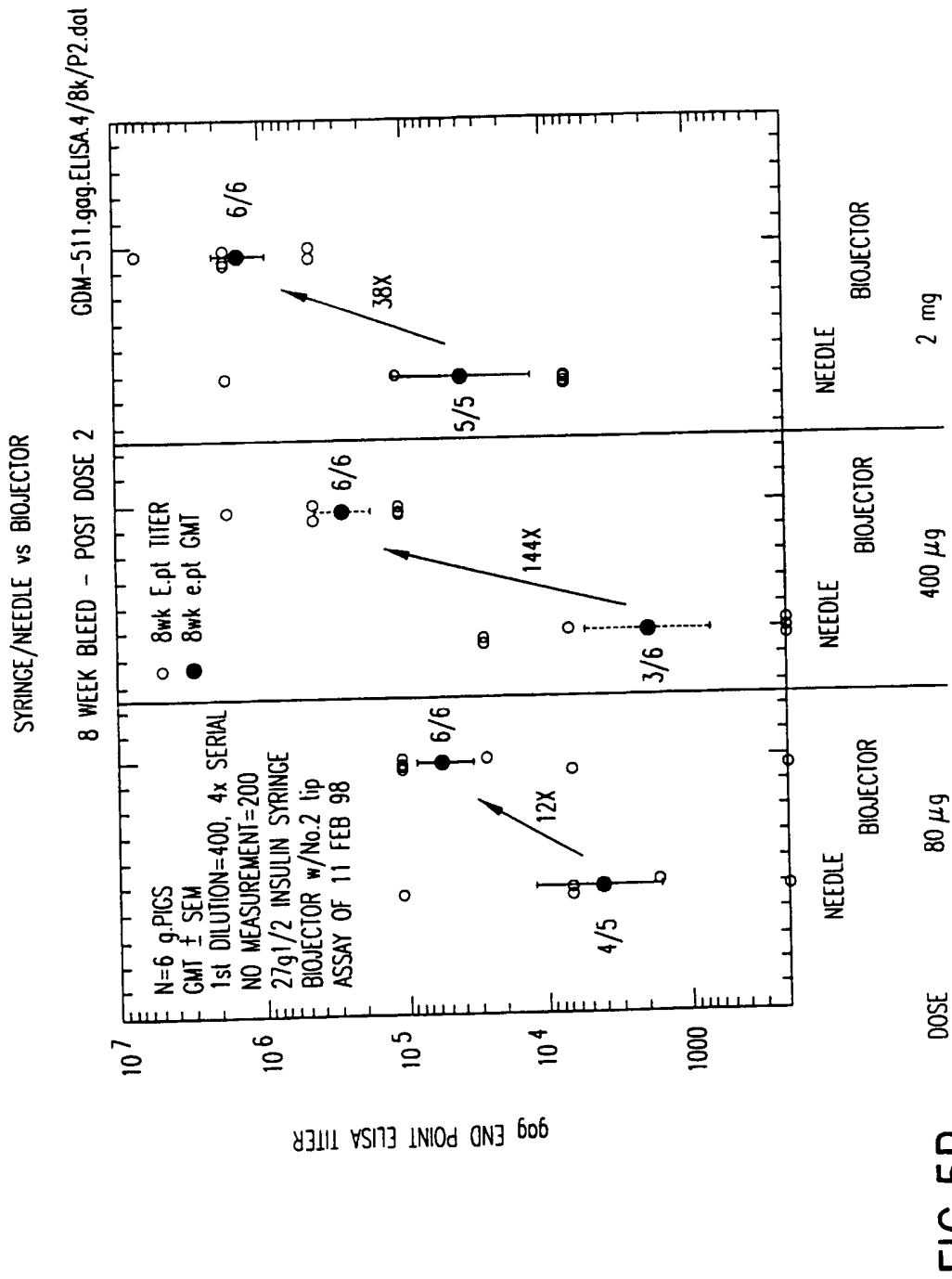

SYRINGE/NEEDLE vs BIOJECTOR
8 WEEK BLEED - POST DOSE 2
GDM-511.gag.ELISA.4/8k/P2.dol
N=6 g.PIGS
GMT ± SEM
1st DILUTION=400, 4x SERIAL
NO MEASUREMENT=200
27g1/2 INSULIN SYRINGE
BIOJECTOR w/No.2 lip
ASSAY OF 11 FEB 98
8wk E.pt TITER
8wk e.pt GMT
gag END POINT ELISA TITER
10^7
10^6
10^5
10^4
1000
6/6
12X
4/5
6/6
144X
3/6
6/6
38X
5/5
NEEDLE
BIOJECTOR
NEEDLE
BIOJECTOR
NEEDLE
BIOJECTOR
DOSE
80 μg
400 μg
2 mg

FIG.5B

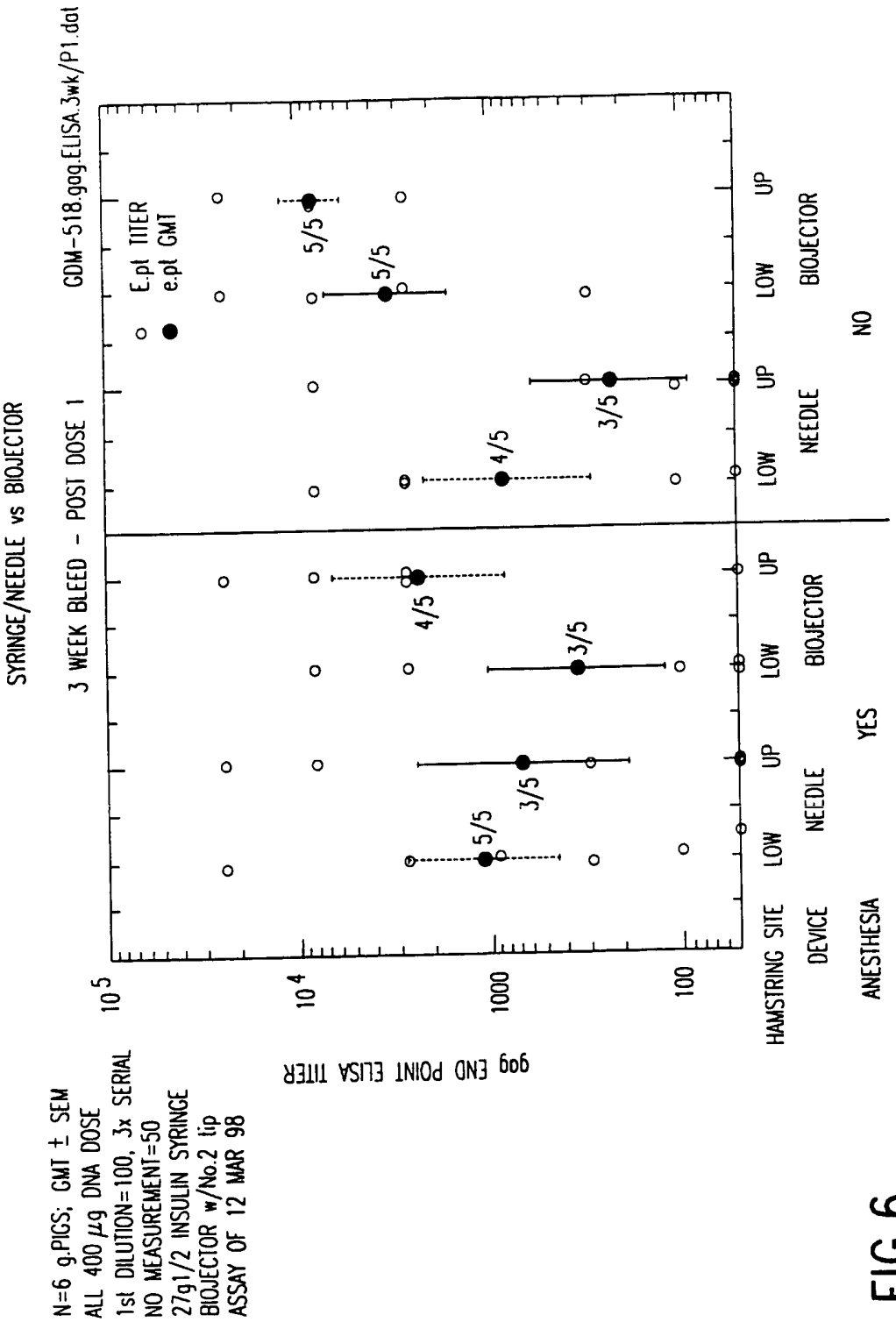

SYRINGE/NEEDLE vs BIOJECTOR
3 WEEK BLEED - POST DOSE 1
GDM-518.gag.ELISA.3wk/P1.dat
E.pl TITER
e.pl GMT
gag END POINT ELISA TITER
10^5
10^4
1000
100
5/5
4/5
3/5
HAMSTRING SITE
DEVICE
ANESTHESIA
LOW
UP
NEEDLE
BIOJECTOR
YES
NO
N=6 g.PIGS; GMT ± SEM
ALL 400 μg DNA DOSE
1st DILUTION=100, 3x SERIAL
NO MEASUREMENT=50
27g1/2 INSULIN SYRINGE
BIOJECTOR w/No.2 tip
ASSAY OF 12 MAR 98

FIG.6

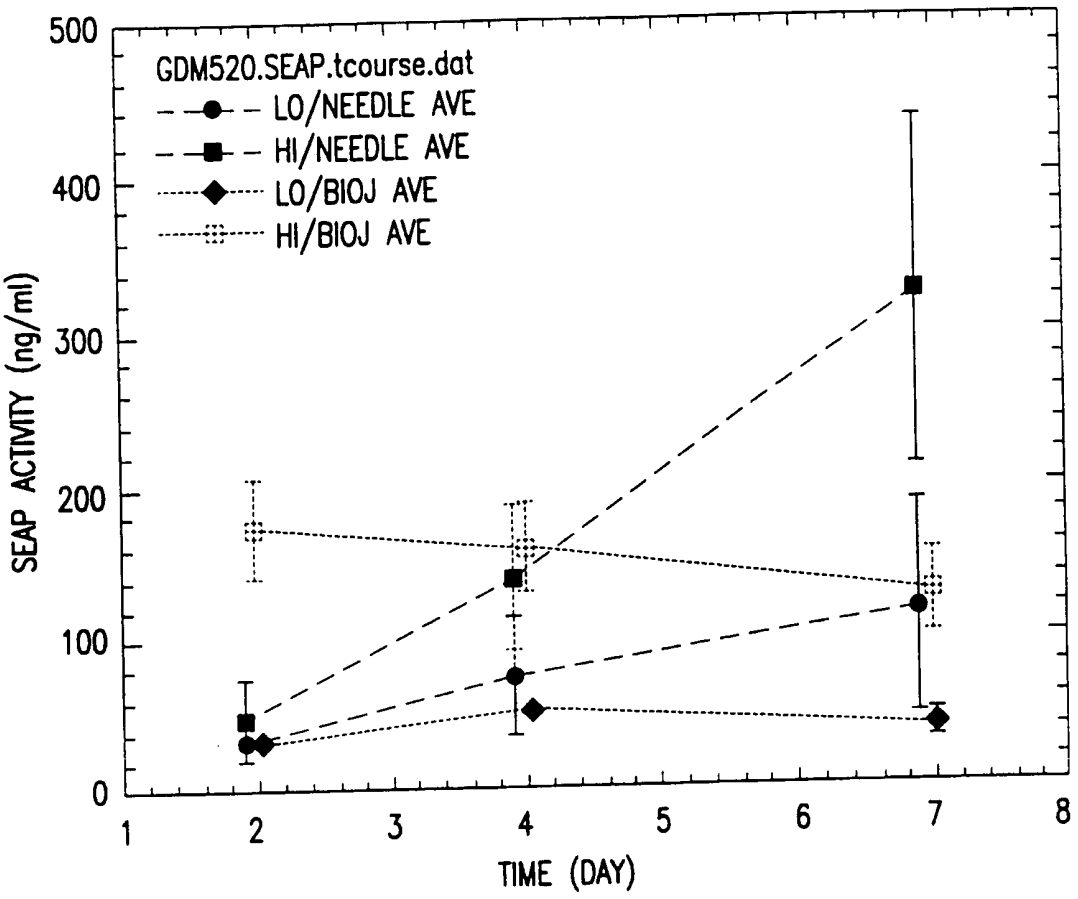

GDM520.SEAP.tcourse.dat
LO/NEEDLE AVE
HI/NEEDLE AVE
LO/BIOJ AVE
HI/BIOJ AVE
SEAP ACTIVITY (ng/ml)
TIME (DAY)
0
100
200
300
400
500
1
2
3
4
5
6
7
8

FIG.7

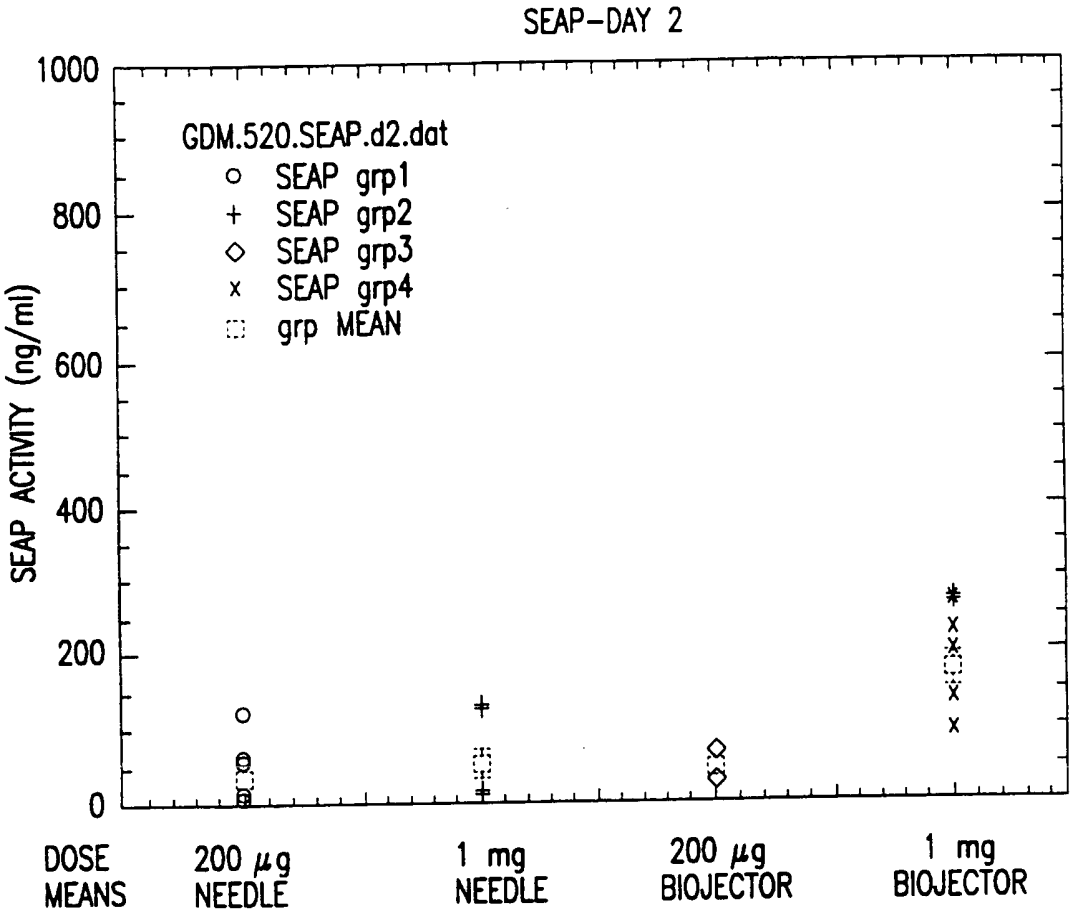

SEAP-DAY 2
GDM.520.SEAP.d2.dat
SEAP grp1
SEAP grp2
SEAP grp3
SEAP grp4
grp MEAN
SEAP ACTIVITY (ng/ml)
1000
800
600
400
200
0
DOSE MEANS
200 µg NEEDLE
1 mg NEEDLE
200 µg BIOJECTOR
1 mg BIOJECTOR

FIG.8A

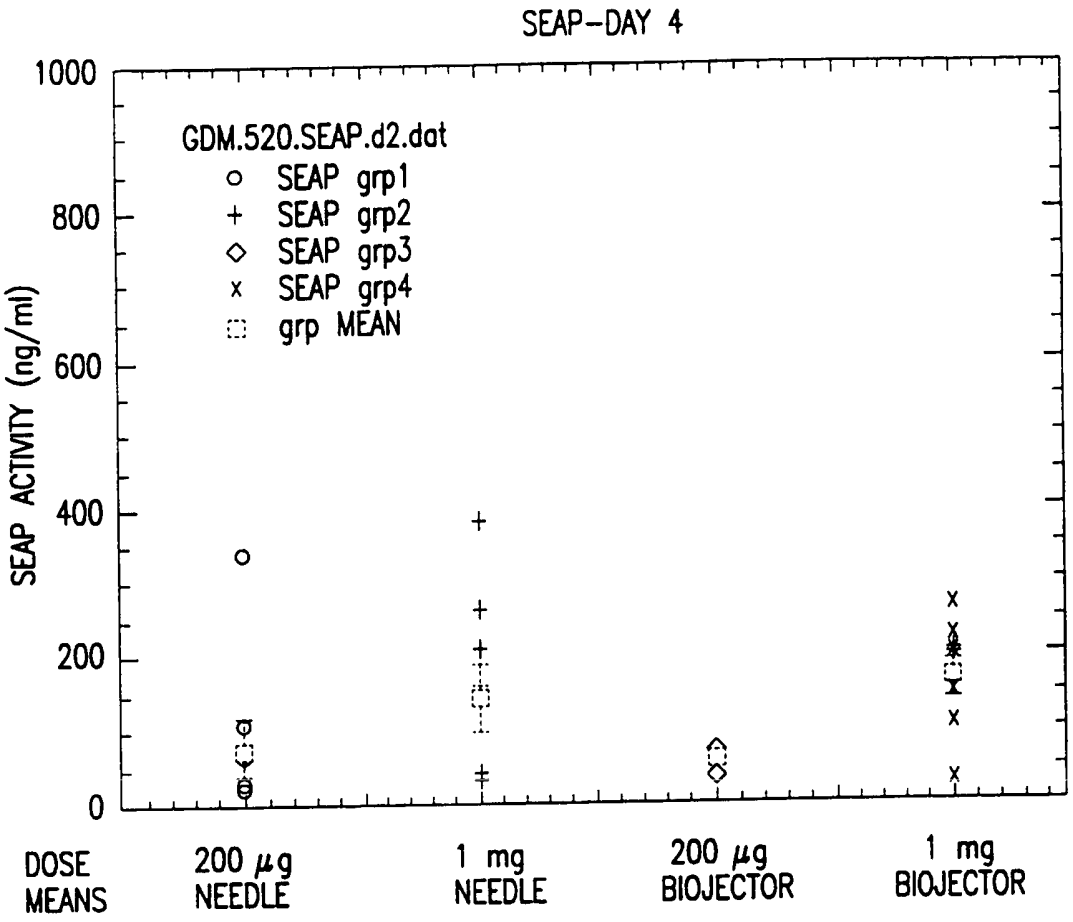

SEAP-DAY 4
GDM.520.SEAP.d2.dat
SEAP grp1
SEAP grp2
SEAP grp3
SEAP grp4
grp MEAN
SEAP ACTIVITY (ng/ml)
1000
800
600
400
200
0
DOSE MEANS
200 µg NEEDLE
1 mg NEEDLE
200 µg BIOJECTOR
1 mg BIOJECTOR

FIG.8B

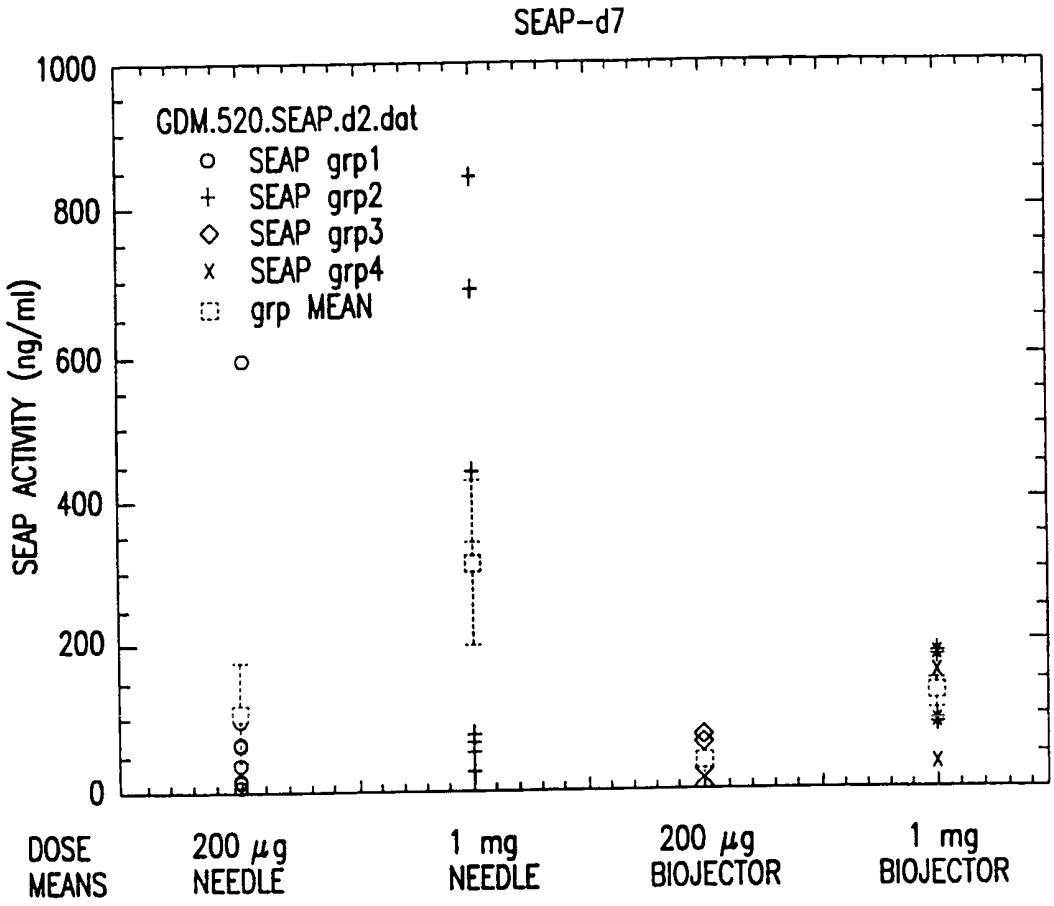

SEAP-d7
GDM.520.SEAP.d2.dat
SEAP grp1
SEAP grp2
SEAP grp3
SEAP grp4
grp MEAN
SEAP ACTIVITY (ng/ml)
1000
800
600
400
200
0
DOSE MEANS
200 µg NEEDLE
1 mg NEEDLE
200 µg BIOJECTOR
1 mg BIOJECTOR

FIG.8C

Viscosity of DNA in Saline at Various Concentrations					
DNA CONCENTRATION	TEMPERATURE	(DYNES/cm^2) SHEAR FORCE	(sec^-1) SHEAR RATE	% S.C.	VISCOSITY AT PEAK SHEAR
25 mcg/ml	+10 C	0-40	2381	84	1.68
	control	0	0	87	
	+25 C	0-28	2384	76	1.18
	control	0	0	85	
	stock			90	
250 mcg/ml	+10 C	0-40	2190	88	1.83
	control	0	0	88	
	+25 C	0-28	2206	87	1.27
	control	0	0	87	
	stock			87	
2500 mcg/ml	+10 C	0-95	2428	86	3.91
	+25 C	0-28	2417	86	2.69
	control	0	0	87	
	stock			86	

Viscosity of DNA in Saline/Glycerol at Various Concentrations					
DNA CONCENTRATION	TEMPERATURE	(DYNES/cm^2) SHEAR FORCE	(sec^-1) SHEAR RATE	% S.C.	VISCOSITY AT PEAK SHEAR
25 mcg/ml	+10 C	0-100	2437	89	4.1
	control	0	0	87	
	+25 C	0-55	2382	90	2.31
	control	0	0	86	
	stock			89	
250 mcg/ml	+10 C	0-100	2514	88	3.98
	control	0	0	89	
	+25 C	0-55	2340	86	2.35
	control	0	0	87	

FIG.9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/07898

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A 61B 19/00
US CL : 128/898

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 128/898; 514/44; 435/6, 69.1, 172.3, 320.1; 604/143, 131, 143, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,736,524 A (CONTENT et al.) 07 April 1998, see entire document.	1-8
A, P	US 5,866,553 A (DONNELLY et al.) 02 February 1999, see entire document.	1-8
A	US 4,940,460 A (CASEY, I et al.) 10 July 1990, see entire document.	1-8
A	US 4,790,824 A (MORROW et al.) 13 December 1988, see entire document.	1-8
A	US 4,596,556 A (MORROW et al.) 24 June 1986, see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 JULY 1999	Date of mailing of the international search report 29 JUL 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KELLY O'HARA Telephone No. (703) 308-0858

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